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(54) Abstract Title
Killing microorganisms with peroxidase under superatmospheric pressure

(57) A method of inactivating or killing microorganisms comprises exposing them to a peroxidase system under superatmospheric pressure. The peroxidase system preferably comprises a peroxidase, an oxidisable peroxidase substrate and hydrogen peroxide. The pressure may be applied prior to addition of the peroxidase system to the microorganisms. The microorganisms may comprise bacteria, yeast or fungi and may be sporulated or non-sporeforming. The peroxidase may be lactoperoxidase, myeloperoxidase, eosinophyl peroxidase or chloroperoxidase. The superatmospheric pressure may be 100-1000 MPa, applied for 1 sec-5 h at -20-100°C. The method may be used to decontaminate, pasteurise or sterilise products or matierals, such as foods, cosmetics, pharmaceuticals or medical equipment. An apparatus for carrying out such a method may comprise sterility test tasks operable to generate and communicate messages to alter pressure generating or peroxidase system operations.

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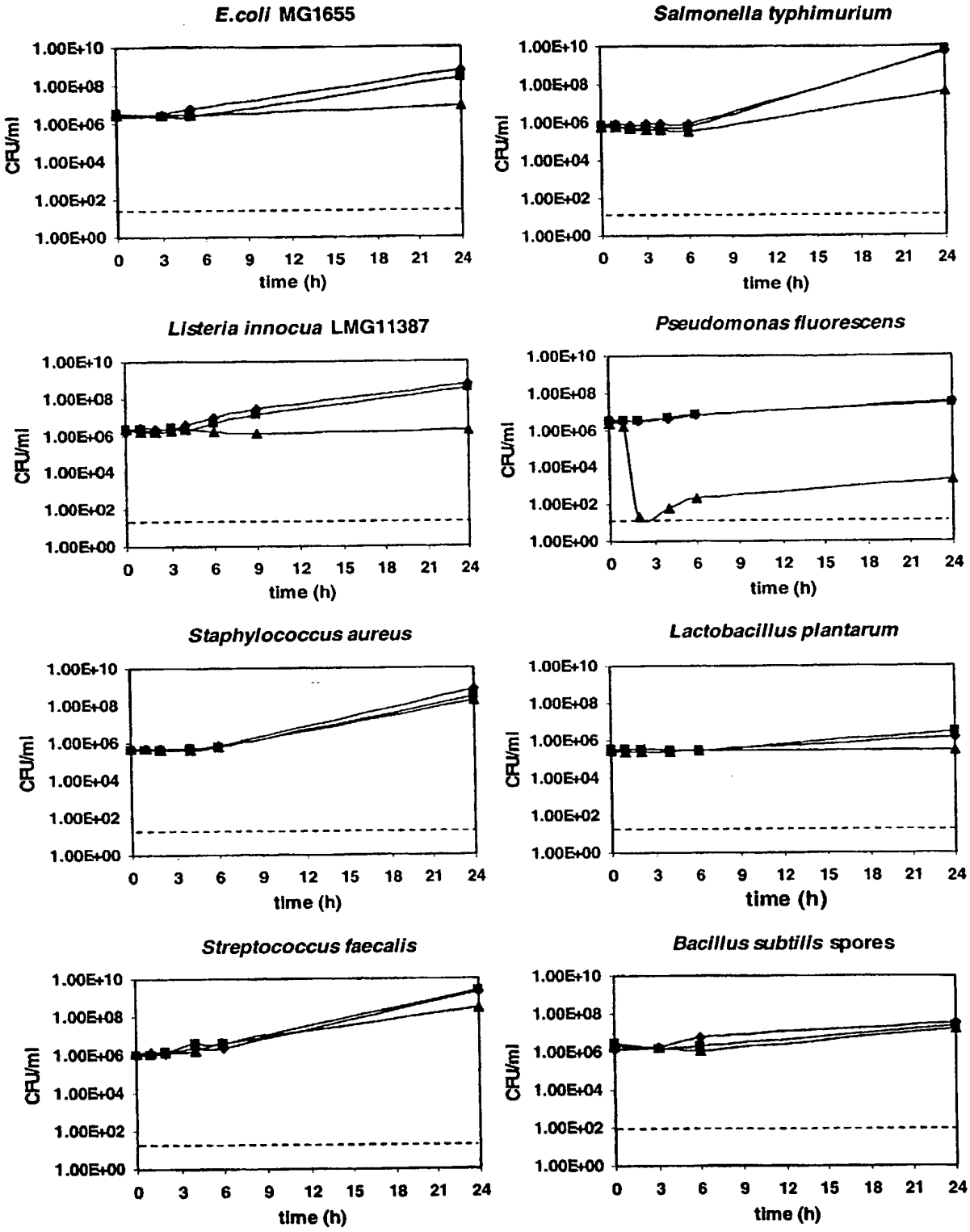
Fig. 1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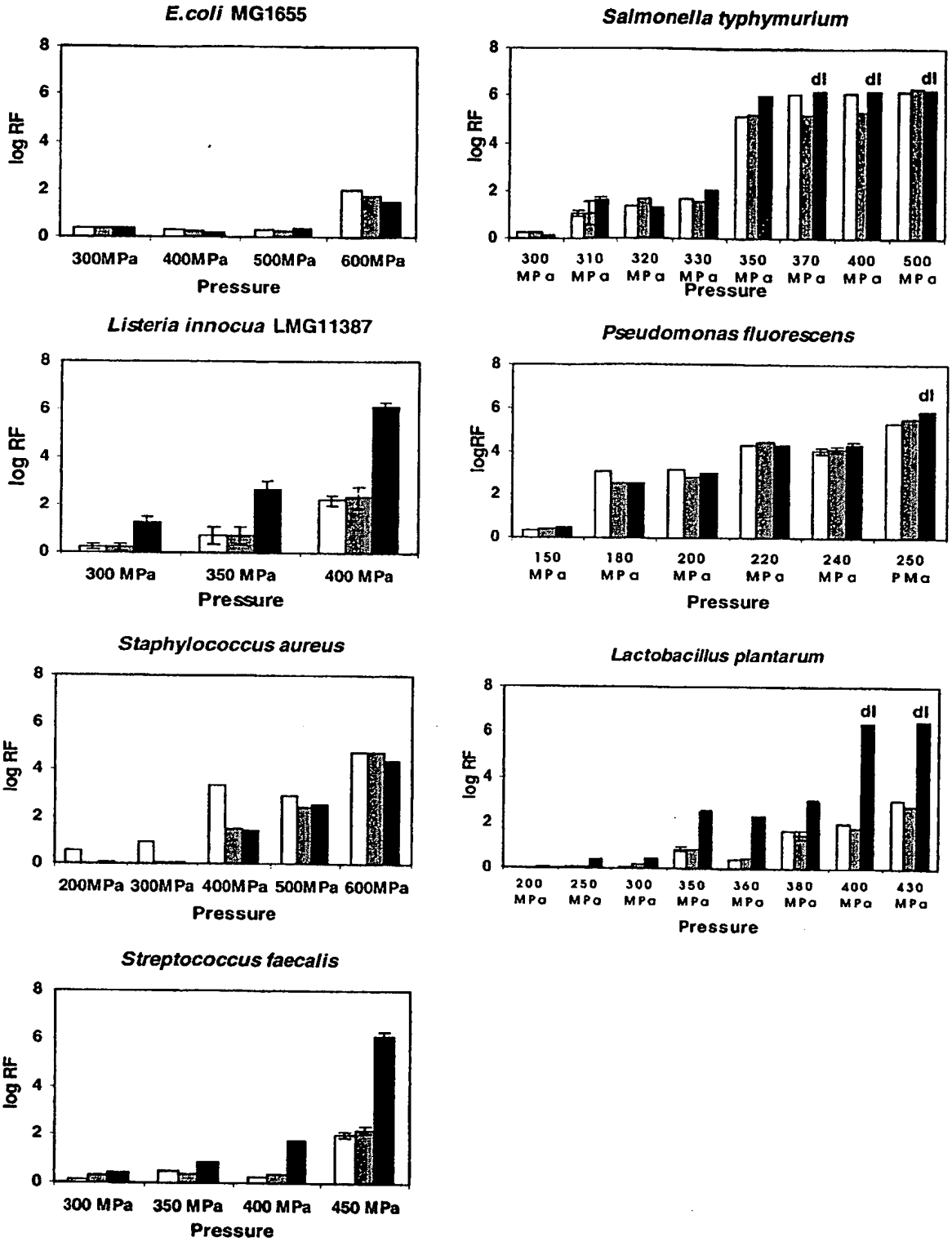
Pseudomonas fluorescens
Staphylococcus aureus
Lactobacillus plantarum
Streptococcus faecalis
Bacillus subtilis spores
CFU/ml
1.00E+10
1.00E+08
1.00E+06
1.00E+04
1.00E+02
1.00E+00
0 3 6 9 12 15 18 21 24
time (h)

Fig. 2


E.coli MG1655
log RF
0
2
4
6
8
300MPa
400MPa
500MPa
600MPa
Pressure
Salmonella typhymurium
dl
dl
dl
300 MPa
310 MPa
320 MPa
330 MPa
350 MPa
370 MPa
400 MPa
500 MPa
Pressure
Listeria innocua LMG11387
300 MPa
350 MPa
400 MPa
Pressure
Pseudomonas fluorescens
logRF
dl
150 MPa
180 MPa
200 MPa
220 MPa
240 MPa
250 PMa
Pressure
Staphylococcus aureus
200MPa
300MPa
400MPa
500MPa
600MPa
Pressure
Lactobacillus plantarum
dl
dl
200 MPa
250 MPa
300 MPa
350 MPa
360 MPa
380 MPa
400 MPa
430 MPa
Pressure
Streptococcus faecalis
300 MPa
350 MPa
400 MPa
450 MPa
Pressure

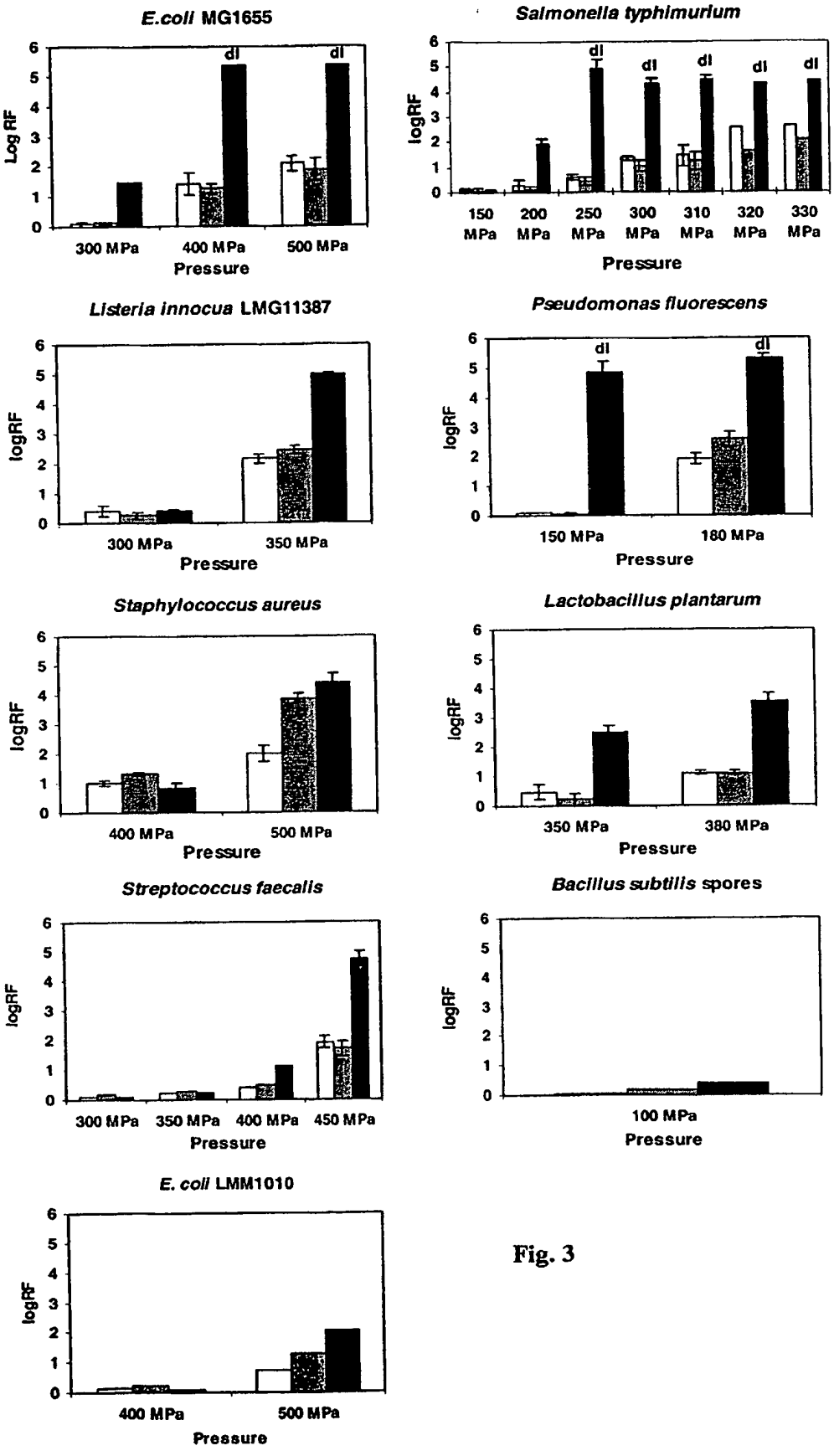

E.coli MG1655
Log RF
dl
300 MPa
400 MPa
500 MPa
Pressure
Salmonella typhimurium
logRF
150 MPa
200 MPa
250 MPa
300 MPa
310 MPa
320 MPa
330 MPa
Listeria innocua LMG11387
300 MPa
350 MPa
Pseudomonas fluorescens
150 MPa
180 MPa
Staphylococcus aureus
400 MPa
500 MPa
Lactobacillus plantarum
350 MPa
380 MPa
Streptococcus faecalis
450 MPa
Bacillus subtilis spores
100 MPa
E. coli LMM1010

Fig. 3

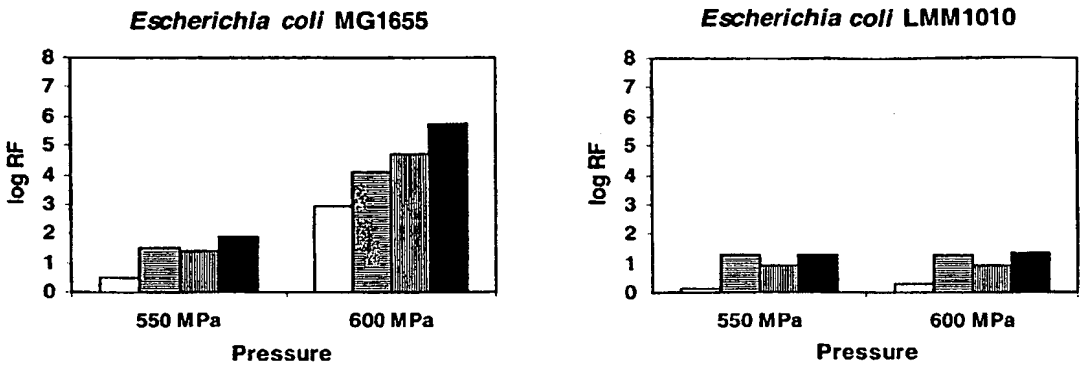

Escherichia coli MG1655
log RF
0
1
2
3
4
5
6
7
8
550 MPa
600 MPa
Pressure
Escherichia coli LMM1010
log RF
0
1
2
3
4
5
6
7
8
550 MPa
600 MPa
Pressure

Figure 4

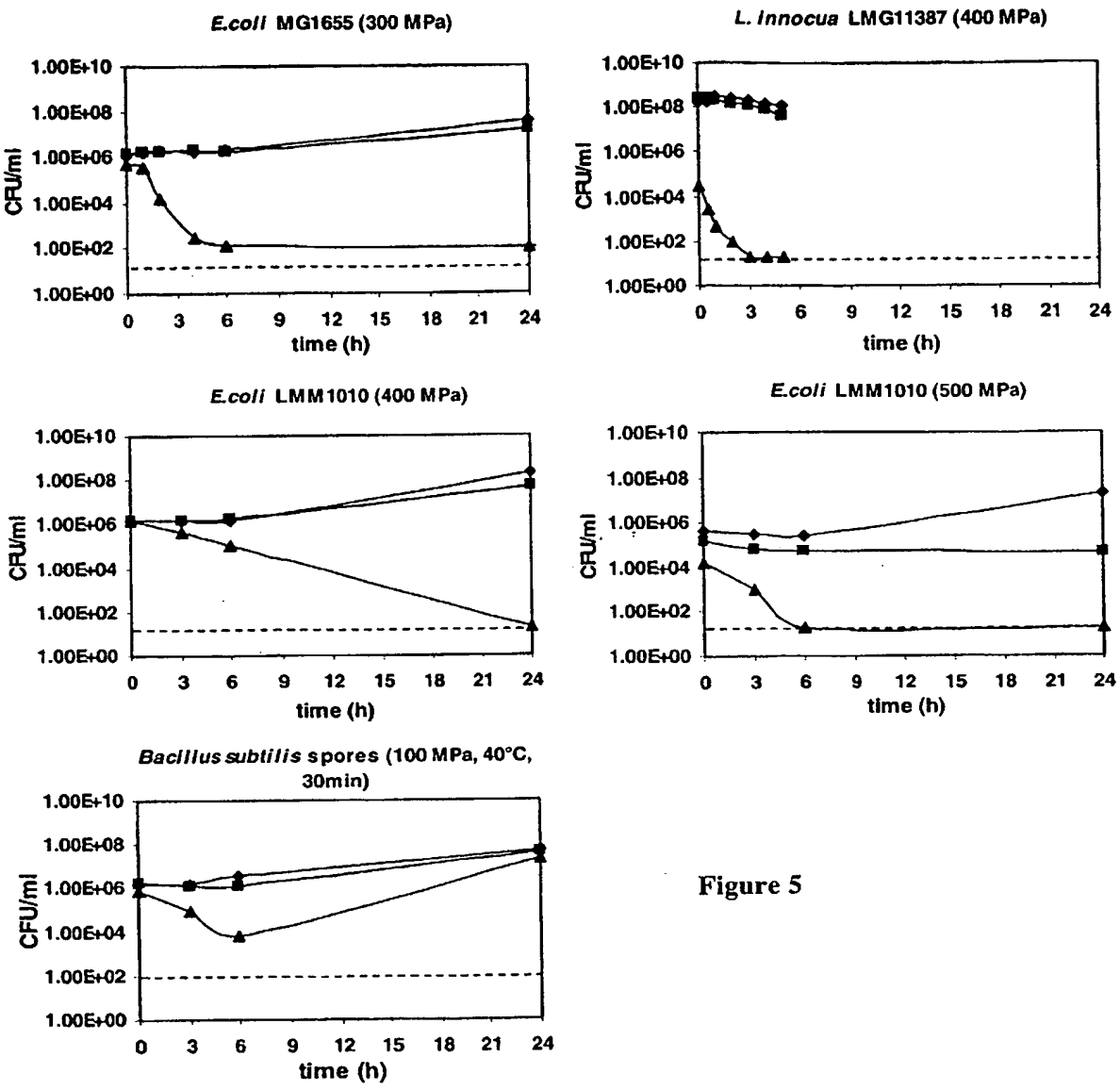
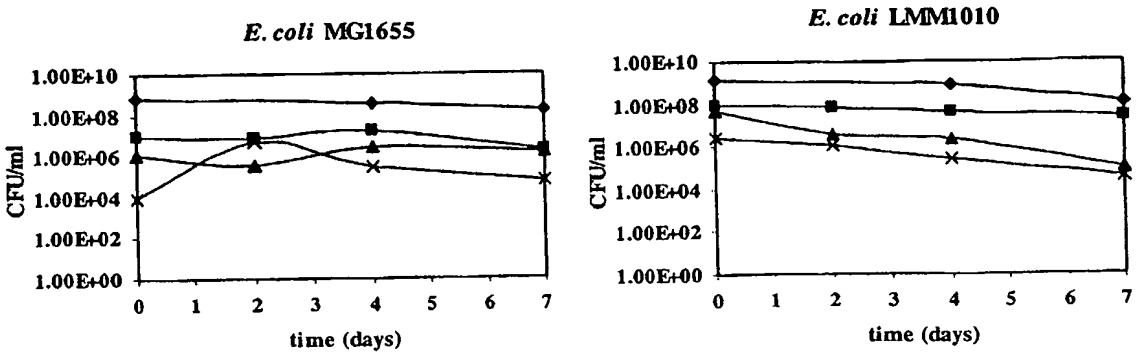
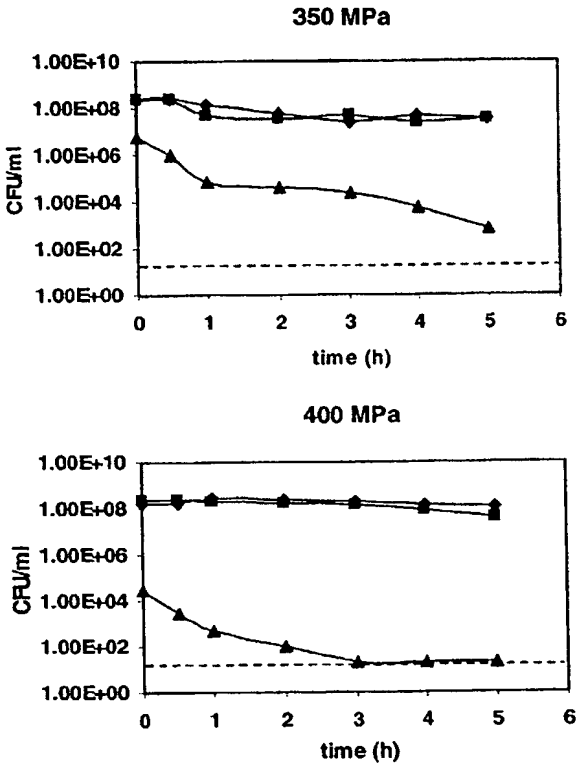

E.coli MG1655 (300 MPa)
L. Innocua LMG11387 (400 MPa)
E.coli LMM1010 (400 MPa)
E.coli LMM1010 (500 MPa)
Bacillus subtilis spores (100 MPa, 40°C, 30min)
CFU/ml
time (h)
1.00E+10
1.00E+08
1.00E+06
1.00E+04
1.00E+02
1.00E+00
0 3 6 9 12 15 18 21 24

Figure 5

Figure 6


E. coli MG1655
1.00E+10
1.00E+08
1.00E+06
1.00E+04
1.00E+02
1.00E+00
CFU/ml
0 1 2 3 4 5 6 7
time (days)
E. coli LMM1010
1.00E+10
1.00E+08
1.00E+06
1.00E+04
1.00E+02
1.00E+00
CFU/ml
0 1 2 3 4 5 6 7
time (days)

Addition of the lactoperoxidase system and H_2O_2 before pressurisation



Addition of the lactoperoxidase system and H_2O_2 after pressurisation

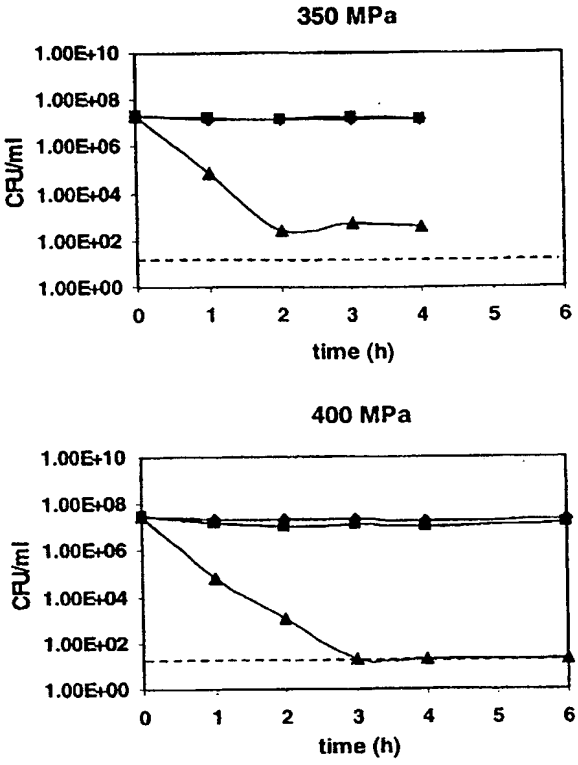


Figure 7

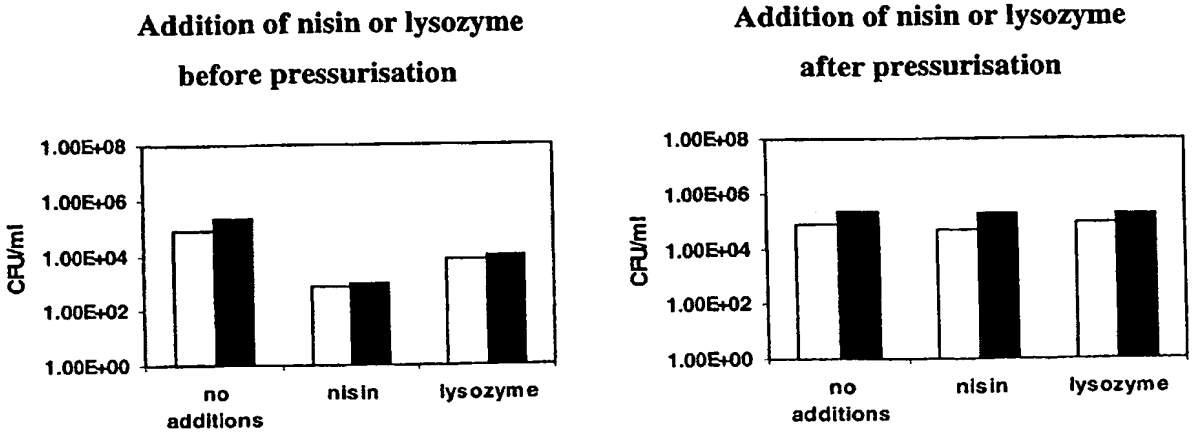

Addition of nisin or lysozyme
before pressurisation
1.00E+08
1.00E+06
1.00E+04
1.00E+02
1.00E+00
CFU/ml
no additions
nisin
lysozyme
Addition of nisin or lysozyme
after pressurisation
1.00E+08
1.00E+06
1.00E+04
1.00E+02
1.00E+00
CFU/ml
no additions
nisin
lysozyme

Figure 8

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IMPROVED COLD PASTEURISATION

FIELD OF THE INVENTION

This invention relates to a method for microbicidal treatment using a combination of superatmospheric pressure and a peroxidase enzyme system. More particularly this method can be applied to decontaminate, disinfect, pasteurise or sterilise products and/or materials in which microbial contamination is a concern.

DESCRIPTION OF THE RELATED ART (BACKGROUND OF THE INVENTION)

Thermal treatment has a long tradition as an industrial process for the inactivation of microorganisms in products such as foods, cosmetics, pharmaceuticals, industrial products, and medical equipment. The intensity of thermal treatment, determined by the temperature and duration of the treatment, determines the level of inactivation that is achieved for each particular microorganism. For instance, to achieve a 10^{12}-fold reduction (12-decimal or 12-D reduction) of spores of *Clostridium botulinum* type A, which is a requirement for sterilisation of low acid foods, a heat treatment of at least 3 min at 121 °C or an equivalent process is required. In general, the microbiological criteria for many pharmaceuticals and medical equipment require high intensity heat treatments similar to the one for sterilised low acid foods. Lower intensity heat treatments find other applications, for instance in the pasteurisation of foods. Pasteurisation is typically carried out at temperatures below 100 °C, and results in less extensive reduction of microorganisms than sterilisation. While pasteurisation efficiently inactivates vegetative bacteria (6-D reduction or higher), some spore-forming microorganisms are not or only marginally inactivated. Therefore, pasteurised foods generally have a strictly limited shelf life and require refrigerated storage. An important problem is that many products/materials are heat sensitive, and suffer from considerable degradation of quality during sterilisation or pasteurisation. Such heat sensitive products can be treated with other, non-thermal techniques such as irradiation, superatmospheric pressure, pulsed electric fields or ultrasonic treatment.

The potential of superatmospheric pressure to extend the shelf-life of fruits, vegetables,

milk and meat had been demonstrated by Hite more than a century ago (Hite 1899, 1914). In spite of promising results, little attention was addressed to the technique at that time presumably because of some major technical difficulties associated with the use of pressure vessels.

Research efforts in the field of pressure preservation were revived since the 80s, initially driven by the idea that pressure processing at ambient or mildly elevated temperatures (*i.e.* cold pressurisation) would allow the production of safe and stable foods. A number of initial reports suggested the potential of superatmospheric pressure to inactivate vegetative microorganisms, and to inhibit undesired enzyme activities without considerable sensorial and nutritional changes in several food products (Cheftel, 1992). Although most research efforts in the field of pressure inactivation of microbes have been performed with the scope of food preservation, the technique can also be used for pasteurisation or sterilisation of other products, such as cosmetics, pharmaceuticals, industrial products, and medical equipment.

However, as the number and variety of studied microorganisms is increasing, the efficiency of cold pressure treatment in the range of conditions that are considered industrially applicable (up to 600 MPa, 0 - 40 °C, 1 - 30 min), to inactivate vegetative bacteria has been questioned by an increasing number of investigators. For instance, it was shown that extremely pressure-resistant variants of *Escherichia coli* can arise after repeated exposure to superatmospheric pressure (Hauben *et al.*, 1997). Very high pressure resistance was later also found among natural *Escherichia coli* strains including pathogenic strains of the O157:H7 serotype (Alpas *et al.*, 1999, Benito *et al.*, 1999). Further, pressure resistance of some bacteria can also increase substantially in some matrices such as milk (García-Graells *et al.*, 1999). Furthermore, within the range of processing conditions that are currently explored for cold pressure processing, sufficient inactivation of microbial spores can not be readily accomplished to meet the 6-D reduction criterion for spores of non-proteolytic *Clostridium botulinum* that exists for some pasteurised foods with extended shelf-life (Maggi *et al.*, 1996, Mills *et al.*, 1998). Only when superatmospheric pressure is combined with a considerably elevated temperature, sufficient inactivation of spores can be achieved (U.S. patent 6 017 572 and U.S. Patent 6 086 936). According to those patents the products are preheated to 75 - 100 °C and then pressurised. It can be anticipated that during the build-up of pressure the product temperature will temporarily further rise to above 100 °C due to

adiabatic heating. Clearly, this technique is not applicable to most heat unstable products. It can be concluded from this brief overview that cold pressure treatment cannot be applied to pasteurise or sterilise non-acid food or other products to meet the existing microbiological criteria for heat pasteurisation or sterilisation, in spite of the potential to inactivate some microorganisms.

A successful application of pressure processing are low pH fruit products (US patent 5 328 703 and WIPO patent WO99/62346). The low pH environment pressure causes an increased reduction of the vegetative microbial flora by superatmospheric pressure, while the combination of chilled storage and acid pH causes further inactivation of vegetative cells that are sublethally injured by the pressure treatment (García-Graells *et al.*, 1998, Linton *et al.*, 1999), and prevents the outgrowth of spores. On the other hand, application of pressure processing for stabilisation of low acid products still awaits further research efforts, to improve inactivation of pressure resistant vegetative cells and spores.

Superatmospheric pressure has been reported by several authors to increase the bactericidal spectrum of lysozyme and some bacteriocins against vegetative bacteria and, vice-versa, these compounds increased the sensitivity of bacteria to pressure inactivation (García-Graells *et al.*, 1999, Hauben *et al.*, 1996, Kalchayanand *et al.*, 1994, Kalchayanand *et al.*, 1998, Masschalck *et al.*, 2000, Ponce *et al.*, 1998). This type of synergy offers an interesting perspective for the development of mild preservation techniques for producing safe and high quality products (Japanese Patents JP3290173 and JP4075574). However, there are still problems. Kinetic experiments clearly revealed that the combined inactivation by superatmospheric pressure and lysozyme and/or nisin is characterised by pronounced tailing, suggesting that a significant fraction of the bacterial population is resistant to the combined treatment (Masschalck *et al.*, 2000). Also, the cooperative effect between pressure and lysozyme or nisin appears to be limited to some bacteria. Several bacteria, including food pathogens such as *Salmonella typhimurium, Salmonella enteritidis,* and *Shigella flexneri* could not be sensitised to lysozyme or nisin by pressure.

Another example of synergistic microbicidal action is the combination of superatmospheric pressure treatment with saccharide esters (United States Patent 6110516). According to this patent, bacteriostatic saccharide esters become

bactericidal against spore-forming bacteria under superatmospheric pressure.

The current patent application concerns the combined application of superatmospheric pressure with a peroxidase enzyme system. Peroxidase enzymes catalyse the oxidation of a suitable substrate by hydrogen peroxide according to the following reaction: [substrate + H(2)O(2) = oxidised substrate + 2 H(2)O]. A well known example is lactoperoxidase, which uses thiocyanate (SCN^-) as a major substrate. Hereafter, we refer to this system shortly as the lactoperoxidase system or LP system.

Lactoperoxidase is a native milk enzyme whose main activity is to catalyse the oxidation of thiocyanate (SCN^-) by hydrogen peroxide into short-lived reactive oxidation products such as the hypothiocyanite anion ($OSCN^-$) that in turn rapidly oxidises many biomolecules. Most relevant for microbial inactivation is probably the oxidation of enzymes and other proteins in the bacterial cell membrane that have exposed sulfhydryl groups (-SH), but other targets have also been suggested. The effectiveness of the lactoperoxidase system depends on different factors, such as the pH and composition of the matrix, the lactoperoxidase concentration, species, the growth stage of microorganisms and the population size. Although bactericidal effects have been observed in some studies and on some microorganisms the lactoperoxidase system cannot be used on its own as a technique for food pasteurisation or sterilisation, since the effect is mostly bacteriostatic when the system is applied at naturally occurring concentrations.

Other peroxidase enzymes such as myeloperoxidase, eosinophyl peroxidase and chloroperoxidase have been described to have antimicrobial effects (US patents 5 510 104 and 6 033 662). These enzymes catalyse the oxidation by H_2O_2 of various substrates such as Cl^-, Br^-, NO_2^- and SCN^- with formation of a variety of reaction products with antimicrobial activity.

Preliminary work on the combination of superatmospheric pressure and lactoperoxidase was conducted in our laboratory on two bacterial species. A strong synergistic effect of pressure and the lactoperoxidase system resulting in efficient inactivation was observed for *Listeria innocua*. However, no synergistic effect was observed for four strains of *Escherichia coli* (García-Graells *et al.*, 1999). It was concluded in this study that some bacteria are sensitised to the lactoperoxidase system by superatmospheric pressure while others are not, and consequently, that a process based on the combined use of superatmospheric pressure and lactoperoxidase can not

replace a thermal pasteurisation because it can not efficiently inactivate all vegetative cells. However in this study the synergistic effect was only evaluated immediately after pressurisation, and a high initial concentration of cells (10^9 CFU/ml) was used. The present invention shows that the inactivation of microbes by the combined process of superatmospheric pressure and the lactoperoxidase system proceeds during storage after pressurisation and that the initial concentration of microbes is critical. When extremely high cell concentrations (*e.g.* 10^9 CFU/ml) are used, as is routinely done in microbial inactivation studies, the combined process of superatmospheric pressure and the lactoperoxidase system shows no synergistic effect for some bacteria. However, we demonstrate in the present invention that microbial inactivation by superatmospheric pressure and the lactoperoxidase system shows a strong synergy when lower cell concentrations are used, which are more likely to occur in a product that has been prepared with proper care of hygiene. Under these conditions, the technique is also effective against the *Escherichia coli*, which was previously reported to be insensitive (García-Graells *et al.*, 1999).

These findings are novel and unexpected because:

(1) It is not generally recognised that cell concentration is critical in inactivation based on superatmospheric pressure alone or on superatmospheric pressure combined with lysozyme or nisin, and hence it was not evident to investigate the effect of superatmospheric pressure combined with the lactoperoxidase system at lower bacterial concentrations.

(2) Inactivation of bacteria after treatment with superatmospheric pressure alone or superatmospheric pressure combined with antimicrobials such as lysozyme or nisin as thus far described is transient, *i.e.* does not continue after release of pressure. Hence it was not evident to investigate the existence of a persistent effect of superatmospheric pressure combined with the lactoperoxidase system during several hours after pressure exposure.

(3) The synergistic effect of superatmospheric pressure and the lactoperoxidase system is observed on a much wider range of different bacteria than is the case for superatmospheric pressure and lysozyme or nisin.

(4) Using the same pressure, much higher levels of inactivation of pressure-resistant bacteria can be achieved in combination with the lactoperoxidase

system than thus far described for combinations with other antimicrobials such as nisin or lysozyme.

DEFINITIONS

The term "Product" used herein, refers anything produced or made either naturally or artificially

The term "Material" used herein, refers that of which something is made or is composed

The term "Peroxidase" used herein, refers to a haem-containing enzyme that uses hydrogen peroxide [H(2)O(2)] as the electron acceptor to oxidise one or more compounds that can be of different chemical structure.

The term "Peroxidase system" used herein, refers to a peroxidase enzyme together with hydrogen peroxide and a compound that can be oxidised by the enzyme, and that, in its oxidised state, has antimicrobial properties

The term "Inactivating" used herein, refers to the reduction in the number of viable cells that occurs when a microbial population is exposed to a certain treatment.

The term "high hydrostatic pressure" or "superatmospheric pressure"used herein, refers to a pressure higher than 50 MPa, that is transmitted by a fluid and used to treat a product or material

The term "decontamination" used herein, refers to a treatment of a product or material intended to reduce the number of viable microorganisms on said product or material.

The term "Sterilisation" used herein, refers to a treatment of a product or material intended to reduce the number of viable microorganisms on said product or material at least with a factor 10exp6

The term "Pasteurisation" used herein, refers to a treatment of a product or material intended to reduce the number of viable pathogenic non-sporulating microorganisms on said product or material

FIGURE LEGENDS

FIGURE 1 shows the evolution of viable counts for eight different bacteria after inoculation in UHT skim milk, without any additives (♦), with 0.25 mM H_2O_2 (■), or with complete lactoperoxidase / SCN^- / H_2O_2 system (▲). Dashed line indicates the detection limit.

FIGURE 2 shows the inactivation (expressed as log RF, *i.e.* log N_0 – log N) of seven different bacteria by superatmospheric pressure treatment alone (□), superatmospheric pressure treatment after addition of 0.25 mM H_2O_2 (▨), Superatmospheric pressure treatment after addition of the lactoperoxidase system (■). Inoculum density 10^9 CFU/ml. Analysis of viable count immediately after pressure treatment. Abbreviation dl: detection limit.

FIGURE 3 shows the inactivation (expressed as log RF, *i.e.* log N_0 – log N) of seven different bacteria by superatmospheric pressure treatment alone (□), superatmospheric pressure treatment after addition of 0.25 mM H_2O_2 (▨), Superatmospheric pressure treatment after addition of the lactoperoxidase system (■). Inoculum density 10^6 CFU/ml. Analysis of viable count immediately after pressure treatment. Abbreviation dl: detection limit.

FIGURE 4 shows the inactivation (expressed as log RF, *i.e.* log N_0 – log N) of *E. coli* MG1655 and LMM1010 by superatmospheric pressure treatment alone (□), superatmospheric pressure treatment after addition of 400 IU/ml nisin (⊟), superatmospheric pressure treatment after addition of 400 µg/ml lysozyme (▣) and superatmospheric pressure treatment after addition of 400 IU/ml nisin and 400 µg/ml

lysozyme (■). Inoculum density 10^9 CFU/ml. Analysis of viable count immediately after pressure treatment.

FIGURE 5 shows the evolution of viable counts of different bacterial suspensions in skim milk after superatmospheric pressure treatment. Samples were kept at 20 °C after pressure treatment. Samples contained either no additions (♦), 0.25 mM H_2O_2 (■), or the complete lactoperoxidase / SCN^- / H_2O_2 system (▲). Dashed line indicates the detection limit.

FIGURE 6 shows the evolution of viable counts of *E. coli* MG1655 or LMM1010 suspensions in skim milk after superatmospheric pressure treatment (550 MPa / 20 °C / 15 min). Samples were kept at 8 °C after pressure treatment. Samples contained either no additions (♦), 400 µg/ml lysozyme (■), 400 IU/ml nisin (▲), or 400 µg/ml lysozyme and 400 IU/ml nisin (X).

FIGURE 7 shows the evolution of viable counts of Listeria innocua in skim milk after pressure treatment. Samples were kept at 20 °C after pressure treatment. Samples contained either no additions (♦), 0.25 mM H_2O_2 (■), or the complete lactoperoxidase / SCN^- / H_2O_2 system (▲).

FIGURE 8 shows the evolution of viable counts of *E. coli* MG1655 in potassium phosphate buffer (10 mM, pH7) 30 min (□) or 165 min (■) after pressure treatment (300 MPa / 20 °C / 15 min). Samples were kept at 20 °C after pressure treatment.

ILLUSTRATIVE EMBODIMENTS OF THE INVENTION

The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention. This invention is not limited to the particular methodology, protocols and reagents described as they may vary.

EXAMPLE 1: The bactericidal effect of the lactoperoxidase system on different microorganisms at atmospheric pressure in UHT skim milk during storage at 20 °C.

The bactericidal effect of the lactoperoxidase system on different microorganisms at atmospheric pressure in UHT skim milk during storage at 20 °C was determined as follows.

To prepare inocula of vegetative bacteria, cultures were grown to stationary phase for 21 h with shaking (200 rpm) at 30 or 37 °C using an appropriate growth medium (Table 1). Cells were harvested by centrifugation (3000 x *g*, 5 min) and resuspended in commercial ultrahigh-temperature-treated (UHT) skim milk at a cell density of approximately 10^6 CFU/ml.

To prepare inocula of bacterial spores, cells from a -80 °C glycerol stock culture were grown at 37 °C in a humid atmosphere on the surface of nutrient agar CM3 (Oxoid, Basingstoke, U.K.) supplemented with 0.06 g/l $MgSO_4$ and 0.25 g/l KH_2PO_4. After seven days, spores were harvested from the plates in sterile demonised water, washed twice and finally resuspended in sterile demonised water to a concentration of 10^7-10^8 spores ml^{-1} and kept at 4 °C. These aqueous spore suspensions were used to inoculate ultrahigh-temperature-treated (UHT) skim milk at a cell density of approximately 10^6 CFU/ml.

Three test samples were made, one with the addition of the complete lactoperoxidase / SCN^- / H_2O_2 system, *i.e.* 5 µg/ml lactoperoxidase (EC 1.11.1.7; Sigma, Bornem, Belgium), 0.25 mM KSCN (Acros, Geel, Belgium) and 0.25 mM H_2O_2 (Vel, Leuven, Belgium), one with addition of 0.25 mM H_2O_2 only, and one without additions. Lactoperoxidase was added from a 10 mg/ml stock solution in 50 % glycerol and 50 % phosphate buffered saline (0.1 M potassium phosphate buffer pH 6.0, 150 mM NaCl). The three test samples were incubated at room temperature during 24 h. At different times the viability of the different samples (CFU/ml) was determined by plating the appropriate decimal dilutions on an appropriate count medium (Table 1), with a spiral plater (Spiral Systems, Cincinnati, Ohio) and incubating at 30 or 37°C for 24 - 48 h. The results are shown in Figure 1.

Table 1: Culture conditions for bacteria used in this example

	Growth medium	Count medium	Growth temperature
Escherichia coli	LB	TSA	37 °C
Listeria innocua	TSB	TSA	37 °C

Staphylococcus aureus	NB	PCA	37 °C
Streptococcus faecalis	BHI	BHI	37 °C
Salmonella typhimurium	NB	PCA	37 °C
Pseudomonas fluorescens	NB	PCA	37 °C
Lactobacillus plantarum	MRS	MRS	30 °C
Bacillus subtilis	NA	TSA	37 °C

Abbreviations: LB (Luria Bertani broth), TSB (trypton soya broth), NB (nutrient broth), BHI (Brain heart infusion broth or agar), MRS (de Man Rogosa Sharpe broth or agar), NA (nutrient agar), TSA (trypton soya agar), PCA (plate count agar).

The example demonstrates that most of the tested microorganisms show total (*E. coli*, *L. innocua*, *L. plantarum*) or partial (*S. typhimurium*, *S. faecalis*) inhibition of growth in milk stored at room temperature by the lactoperoxidase system (5 µg/ml lactoperoxidase, 0.25 mM KSCN and 0.25 mM H_2O_2). However, *P. fluorescens* appears to be very sensitive to the lactoperoxidase system and shows strong and rapid reduction in viability. *S. aureus* and *B. subtilis*, on the other hand are not inhibited at all by the lactoperoxidase system under the conditions of the experiment.
It can be concluded that the lactoperoxidase system, when applied at a concentration as it occurs naturally in milk, does not inactivate (i.e. kill) the majority of bacteria, but retards growth of some bacteria. The lactoperoxidase system in its own can thus not be used as a technique for cold pasteurisation.

EXAMPLE 2: The bactericidal effect of the lactoperoxidase system under superatmospheric pressure on highly concentrated cell suspensions (10^9 CFU/ml), evaluated immediately after the pressure treatment

The bacterial suspensions are prepared as in example 1, except that a cell concentration of 10^9 CFU/ml was used instead of 10^6 CFU/ml. Samples in heat-sealed polyethylene bags were pressurised in a small 8-ml pressure autoclave driven by a manual spindle pump and thermostatically controlled with a water jacket connected to a cryostat (Resato, Roden, The Netherlands). The pressure liquid was a mixture of water and glycol. The compression and decompression rates were 100 MPa/min and 300 MPa/min respectively. All pressure treatments were conducted at 20°C during 15 min, except when otherwise mentioned. It should be noted however that sample temperatures in our experiments may temporarily increase during adiabatic

compression. Measurements with thermocouples fitted in the pressure vessel, indicated a maximum temperature at 600 MPa of 38 °C, using the same compression rate as used here.

Viability of pressure-treated bacteria and untreated controls was determined as in example 1. Reduction of viable cells (log RF) was expressed as the difference between the logarithms of the colony counts of the untreated and treated samples (log No - log N). The results are shown in Figure 2.

This example demonstrates that the presence of an active lactoperoxidase system strongly increases the inactivation by superatmospheric pressure of *L. innocua, L. plantarum* and *S. faecalis*, all at an initial inoculum density of 10^9 CFU/ml. Since these bacteria were not inactivated by the lactoperoxidase system in the absence of pressure treatment, it can be concluded that superatmospheric pressure and the lactoperoxidase system work synergistically on these bacteria. For the other tested bacteria, *E. coli, S. typhymurium, P. fluoresens* and *S. aureus*, no such synergy occurred.

Based on the results in this example, use of superatmospheric pressure in combination with the lactoperoxidase system could not be advocated as an effective technique for cold pasteurisation, because even using pressures up to 600 MPa, some vegetative bacteria (e.g. *E. coli* MG1655) are insufficiently inactivated (i.e. less than 6-D)

EXAMPLE 3: The bactericidal effect of the lactoperoxidase system under superatmospheric pressure on moderately concentrated cell suspensions (10^6 CFU/ml), evaluated immediately after the pressure treatment

The bactericidal effect of the combined treatment by the lactoperoxidase system and superatmospheric pressure on moderately concentrated bacterial suspensions was determined as in example 2, except that a cell concentration of 10^6 CFU/ml was used instead of 10^9 CFU/ml. The results are shown in Figure 3.

This example demonstrates that, if moderately concentrated cell suspensions (10^6 CFU/ml) were used, higher levels of inactivation were achieved by superatmospheric pressure treatment of all tested vegetative bacteria in the presence of the lactoperoxidase system than in its absence. Spores of *B. subtilis*, in contrast, were not

inactivated by a pressure treatment (100 MPa) in presence of the lactoperoxidase system. It can be concluded that the lactoperoxidase system is an effective means to increase the levels of inactivation of vegetative bacteria that can be achieved by superatmospheric pressure treatment. For highly pressure resistant vegetative cells however, such as *E. coli* LMM1010, the levels of inactivation that can be achieved, even at superatmospheric pressure (500 MPa), are insufficient (less than 6-D) to permit the use of a combined treatment by superatmospheric pressure and the lactoperoxidase system as a cold pasteurisation technique.

EXAMPLE 4: The bactericidal effect of antimicrobial peptides such as lysozyme and nisin under superatmospheric pressure in UHT skim milk

Bacterial suspensions were prepared as in example 1, except that a cell concentration of 10^9 CFU/ml was used. Four test sample were made, one with addition of 400 IU/ml nisin, one with addition of 400 μg/ml lysozyme, one with addition of 400 μg/ml lysozyme and 400 IU/ml nisin, and one without additions. Nisin was added from a 10^4-IU/ml stock suspension of Nisaplin (Aplin & Barret, Trowbridge, UK) in 0.02 N HCl stored at 4 °C. A stock solution of 2 mg/ml of hen egg-white lysozyme (Fluka, Buchs, Switzerland) was prepared in distilled water and frozen in small proportions until use. The samples were pressurised as in example 2. The viability (CFU/ml) of pressure-treated samples and untreated controls was determined. Reduction of viable cells (log RF) under the different conditions are shown in Figure 4.

This example demonstrates that the addition of nisin and/or lysozyme increases the inactivation of *E. coli* under superatmospheric pressure. However, even at superatmospheric pressure (600 MPa), insufficient reduction (less than 6-D) of the most pressure-resistant strain is achieved to permit use of this combined treatment as a cold pasteurisation technique.

EXAMPLE 5: Persistent bactericidal effect of the lactoperoxidase system after pressurisation.

The bacterial suspensions were prepared and pressurised as in example 3. After pressurisation the samples were stored at room temperature and bacterial viability (CFU/ml) was determined at different times after pressurisation. The results are shown in Figure 5.

This example demonstrates that viable counts of all tested bacteria continued to decrease during storage at room temperature after pressure treatment when the lactoperoxidase system was present, while this was not the case when no additives or only H_2O_2 was present in the samples. Since the lactoperoxidase system had no bactericidal activity on these bacteria at atmospheric pressure (see example 1), it appears that the pressure treatment has sensitised the bacterial cells to the lactoperoxidase system, and that the bacteria remain sensitised after release of pressure. Also, the example shows that the lactoperoxidase system is sufficiently pressure stable to preserve its bactericidal activity after pressure treatment.
The levels of inactivation that are achieved for vegetative bacteria in this example, 6 to 24 h after pressure treatment, are much higher than in example 3, where survival was determined immediately after pressure treatment. Even the most pressure resistant strain (*E. coli* LMM1010) is inactivated to below detection limit (> 5-D reduction). This example shows that combined treatment by superatmospheric pressure and the lactoperoxidase system can be used as a technique for cold pasteurisation that can achieve a sufficient reduction of all the vegetative bacteria.
In addition, this example also shows that a more limited inactivation of bacterial spores is also possible with combined technique: between 0 and 6 hours after pressure treatment of a *B. subtilis* spore suspension in the presence of the lactoperoxidase system, the number of viable cells decreased more than 2-D.

A preferred embodiment of the invention is thus a method of combined pressure and peroxidase system pre-treatment of micro-organisms to render the micro-organisms more susceptible to subsequent inactivation by the already present peroxidase system.

EXAMPLE 6: Lack of persistent bactericidal effect of antimicrobial peptides such as lysozyme and nisin after pressurisation

The bacterial suspensions were prepared and pressurised as in example 4. After pressurisation the samples were stored at 8 °C and bacterial viability (CFU/ml) was determined at different times after pressurisation. The results are shown in Figure 6

This example demonstrates that as opposed to the lactoperoxidase system (see example 5), nisin or lysozyme do not cause further inactivation during storage at 8 °C after pressurisation. The bactericidal effect of nisin and lysozyme in combination with superatmospheric pressure is transient.

EXAMPLE 7: Addition of the lactoperoxidase system before or after pressurisation

The effect of adding the lactoperoxidase system before or after pressurisation was investigated on *Listeria innocua*. Two sets of samples were prepared. One set was prepared, pressurised and evaluated as in example 5. In the other set, the addition of the additives took place after the pressurisation instead of before. The results are shown in Figure 7.

This example demonstrates that addition of the lactoperoxidase system after superatmospheric pressure treatment also results in inactivation of pressure treated bacteria. Comparable levels of inactivation are achieved as in the case where the lactoperoxidase system is added before superatmospheric pressure treatment.

EXAMPLE 8: Addition of nisin or lysozyme before or after pressurisation

A culture of *E. coli* MG1655 was grown to stationary phase for 21 h with shaking (200 rpm) at 37 °C using Luria Bertani broth. Cells were harvested by centrifugation (3000 x *g*, 5 min) and resuspended in potassium phosphate buffer (10 mM, pH 7) at a cell density of approximately 10^9 CFU/ml. Two sets of samples were prepared. In one set the addition of nisin (100 IU/ml) or lysozyme (10 µg/ml) took place before the pressurisation, while in the other set, the addition of nisin or lysozyme took place after the pressurisation. Samples in heat-sealed polyethylene bags were pressurised to 300 MPa at room temperature during 15 min in a pressure vessel (National Forge Europe, Belgium) consisted of a stainless-steel cylinder (inner dimensions 50 by 300 mm)

filled with an oil-water emulsion which was compressed by a hydraulic pump. After the pressurisation the samples were stored at room temperature and bacterial viability (CFU/ml) was determined 30 and 165 min after pressurisation. The results are shown in Figure 8.

This example demonstrates that addition of nisin or lysozyme after pressure treatment does not cause further inactivation of pressure-treated cells. Sensitisation to these compounds by superatmospheric pressure is transient.

Another preferred embodiment of the invention is thus a method of pressure pre-treatment of microorganisms to render the microorganisms more susceptible to inactivation by further treatment by a peroxidase system.

DETAILED DESCRIPTION

This invention involves the combination of a peroxidase enzyme system with a superatmospheric pressure treatment. In its preferred embodiment the peroxidase enzyme system involves the use of a peroxidase enzyme system comprsing following three components, in a ratio sufficient to evoke an oxidation reaction, which can be added to the product or material to be treated either before pressure treatment or shortly after pressure treatment, preferably within 1 s – 24 hours after pressure treatment:

a) a peroxidase enzyme such as lactoperoxidase, myeloperoxidase, eosinophyl peroxidase, horseradish peroxidase, soybean peroxidase etc., in a ratio sufficient to catalyse the oxidation reaction.
b) hydrogen peroxide, or a source of hydrogen peroxide such as an oxidase enzyme such as glucose oxidase or xanthine oxidase etc., in a ratio sufficient to oxidise a suitable substrate.
c) a suitable substrate that can be oxidised according to the reaction [substrate + H(2)O(2) = oxidised substrate + 2 H(2)O], catalysed by the peroxidase enzyme in question. Although each specific peroxidase enzyme can have its particular substrate specificity, suitable substrates include but are not limited to thiocyanate, halides such as chloride or iodide, nitrite, etc. These substrates can be naturally

present in the product to be treated or can be added to a concentration that results in the formation of sufficient oxidised product to exert a synergistic microbicidal effect together with pressure treatment.

One or more components of the peroxidase enzyme system can also be naturally present in the product to be treated.

In its preferred embodiment the superatmospheric pressure treatment consists of exposure during between 1 s and 60 min to a pressure of between 100 and 1000 MPa. Pressure treatment can be conducted either as a single exposure, or as a series of multiple exposures with short intervals at a pressure below 100 MPa and lasting between 1 s and 60 min. Pressure treatment is conducted at a temperature below the temperature required for thermal inactivation, preferably between –20°C and +50°C for inactivation of vegetative cells, and between –20°C and +100°C for heat-resistant spores.

3. The post-pressure-treatment holding

After treatment with superatmospheric pressure the treated product containing the peroxidase enzyme system is subjected to a holding period of between 0 and 72 hours at a temperature preferably between 0°C and 50°C, during which further inactivation of the pressure-treated microorganisms by the peroxidase enzyme system will take place.

This invention involves a highly efficient microbicidal treatment that comprises a combined treatment with superatmospheric pressure and with a peroxidase enzyme system, without the necessity to use heat. Superatmospheric pressure is known to have a microbicidal effect, but unless the pressure treatment is conducted at elevated temperatures above about 45 degree °C, extremely superatmospheric pressures are required to kill particular types of microorganisms including bacterial spores and some vegetative (i.e. non-sporulating) bacteria, and this limits the practical usefulness of cold superatmospheric pressure treatment for the purpose of disinfection, decontamination, pasteurisation or sterilisation. On the other hand peroxidase enzyme systems such as the lactoperoxidase system are known to have mainly a microbiostatic effect, not a microbicidal effect. The process according to the present invention of combining pressure treatment with exposure to a peroxidase system allows high levels

of inactivation of all types of vegetative microorganisms, including those that are highly resistant to superatmospheric pressure treatment alone including but not limited to Escherichia coli LMM1010 and Escherichia coli strain O157:H7, without the use of elevated temperatures, and using pressures that are much lower than those required when only superatmospheric pressure is used. The combined process is also more efficient in killing bacterial spores, including but not limited to *Bacillus subtilus* spores *Clostridium sporogenes* and *Clostridium botulinum* spores, than treatment with either factor alone.

Combined treatment of superatmospheric pressure in the presence of particular microbicidal or microbiostatic compounds has been reported before. The applicability of these treatments was limited because they did not allow sufficiently high levels of inactivation, and/or some pressure-resistant microorganisms were not affected.

The present invention unexpectedly demonstrated to be more efficient and active on a much wider range of organisms than combinations of superatmospheric pressure and antimicrobial compounds from prior art. Because present invention improves the efficiency of cold pressure inactivation of microorganisms, it allows the use of much lower pressures for the purpose of desinfection, decontamination, pasteurisation and/or sterilisation than the currently available technology. A unique feature and advantage that distinguishes present invention from other processes based on the combination of superatmospheric pressure and antimicrobial compounds, is that the antimicrobial peroxidase system used in our invention continues to inactivate the microorganisms even after the pressure treatment has been stopped, although the peroxidase enzyme system alone has only a microbiostatic affect on microorganisms that have not been subjected to pressure treatment.

SUMMARY OF THE INVENTION

It is an object of present invention to provide a method for killing microorganisms, characterised in that said microorganisms are exposed to superatmospheric pressure in the presence of a peroxidase system comprising a peroxidase enzyme, a hydrogen peroxide and an oxidisable substrate for said peroxidase. In a second preferred embodiment of present invention the method for killing microorganisms is characterised in that said microorganisms are exposed first to superatmospheric

pressure, and subsequently to a peroxidase system comprising a peroxidase enzyme , a hydrogen peroxide and an oxidisable substrate for said peroxidase, or a method as above but wherein hydrogen peroxide is not added directly but generated in situ, for example by an oxidase enzyme such as glucose oxidase. The microorganisms can be from the group consisting of bacteria, yeasts and fungi

REFERENCES

1. **Alpas, H., N. Kalchayanand, F. Bozoglu, A. Sikes, C. P. Dunne, and B. Ray**. 1999. Variation in resistance to hydrostatic pressure among strains of food-borne pathogens. Appl. Environ. Microbiol. **65**:4248-4251.
2. **Benito, A., G. Ventoura, M. Casadei, T. Robinson, and B. Mackey**. 1999. Variation in resistance of natural isolates of *Escherichia coli* O157 to high hydrostatic pressure, mild heat, and other stresses. Appl. Environ. Microbiol. **65**:1564-1569.
3. **Cheftel, J.C.** 1992. Effects of high hydrostatic pressure on food constituents: an overview. In: High Pressure and Biotechnology. Balny, C., Hayashi, R., Heremans, K., and Masson, P. (eds.) Colloque INSERM/John Libbey Eurotext, Montrouge, France, pp. 195-209.
4. **García-Graells, C., K.J.A. Hauben, and C. W. Michiels.** 1998. High-pressure inactivation and sublethal injury of pressure-resistant *Escherichia coli* mutants in fruit juices. Appl. Environ. Microbiol. **64**:1566-1568.
5. **García-Graells, C., B. Masschalck, and C. W. Michiels.** 1999. Inactivation of *Escherichia coli* in milk by high-hydrostatic-pressure treatment in combination with antimicrobial peptides. J. Food Prot. **62**:1248-1254.

6. **Hauben, K. J. A., E. Y. Wuytack, C. C. F. Soontjens, and C. W. Michiels.** 1996. High-pressure transient sensitisation of *Escherichia coli* to lysozyme and nisin by disruption of outer-membrane permeability. J. Food Prot. **59**:350-355.

7. **Hauben, K. J. A, D. H. Barlett, C. C. F. Soontjes, K. Cornelis, E. Y. Wuytack, and C. W. Michiels.** 1997. *Escherichia coli* mutants resistant to inactivation by high hydrostatic pressure. Appl. Environ. Microbiol. **63**:945-950.

8. **Hite, B.H.** 1899. The effect of pressure in the preservation of milk. Bulletin of the West Virginia University Agricultural Experiment Station **58**:15-35.

9. **Hite, B.H., N.J. Giddings, and C.E. Weakley.** 1914. The effect of pressure on certain microorganisms encountered in the preservation of fruits and vegetables. Bulletin of the West Virginia University Agricultural Experiment Station **146**:1-67.

10. **Kalchayanand, N., T. Sikes, C. P. Dunne, and B. Ray.** 1994. Hydrostatic pressure and electroporation have increased bactericidal efficiency in combination with bacteriocins. Appl. Environ. Microbiol. **60** 4174-4177.

11. **Kalchayanand, N., A. Sikes, C. P. Dunne, and B. Ray.** 1998. Interaction of hydrostatic pressure, time and temperature of pressurization and pediocin AcH on inactivation of foodborne bacteria. J. Food Prot. **61**:425-431.

12. **Linton M., J.M. McClements, M.F. Patterson.** 1999. Survival of *Escherichia coli* O157:H7 during storage in pressure-treated orange juice. J. Food Protect **62**:1038-1040.

13. **Maggi, A., S.Gola, P. Rovere, L. Miglioli, G. Dall'Aglio, and N.G. Lönneborg** 1996. Effetto di trattamenti combinati alta pressione-temperatura su spore di *Clostridium sporogenes* in substrati liquidi. Industria Conserva **71**:8-14

14. **Masschalck, B., C. García-Graells, E. Van Haver, and C. W. Michiels.** 2000. Inactivation of high pressure resistant *Escherichia coli* by lysozyme and nisin

under high pressure. Innovative Food Science and Emerging Technologies. **1**:39-48.

15. **Mills, G., R.arnshaw, and M.F. Patterson** 1998. Effects of high hydrostatic pressure on *Clostridium sporogenes* spores. Letters Appl. Microbiol. **26**:227-230.

16. **Ponce, E., R. Pla, E. Sendra, B. Guamis, and M. Mor-Mur.** 1998. Combined effect of nisin and high hydrostatic pressure on destruction of *Listeria innocua* and *Escherichia coli* in liquid whole egg. Int. J. Microbiol. **43**:15-19.

Further references: U.S. patent 6 017 572; U.S. Patent 6 086 936; US patent 5 328 703; WIPO patent WO99/62346; Japanese Patent JP3290173; Japanese patent JP4075574; United States Patent 6110516; US patent 5 510 104US Patent 6 033 662.

CLAIMS

1. A method of inactivating or killing microorganisms, comprising: exposing said microorganisms to a superatmospheric pressure and exposing said microorganisms to components of a peroxidase system, which comprises a peroxidase, an oxidisable peroxidase substrate and hydrogen peroxide.
2. The method as claimed in claim 1, wherein said microorganisms are first exposed to said superatmospheric pressure and then to said peroxidase system.
3. The method as claimed in claim 1 or 2, wherein said microorganisms are from the group consisting of bacteria, yeasts and fungi.
4. The method as claimed in claim 1 or 2, wherein said microorganisms are non-sporulated or non-sporeforming organisms or mixtures thereof.
5. The method as claimed in claim 1 or 2, wherein the components of said peroxidase system comprises an oxidase, that produces hydrogen peroxide in the presence of a suitable substrate for said oxidase.
6. The method as claimed in claim 1 or 2, wherein: the peroxidase system is a lactoperoxidase system.
7. The method as claimed in claim 1 or 2, wherein: the peroxidase system is a myeloperoxidase system.
8. The method as claimed in claim 1 or 2, wherein: the peroxidase system is a eosinophyl peroxidase system.
9. The method as claimed in claim 1 or 2, wherein: the peroxidase system is a chloroperoxidase system.
10. The method as claimed in claim 1 or 2, wherein the level of superatmospheric pressure is between 100 and 1000 MPa, the exposure time to superatmospheric pressure is between 1 second and 5 hours, and the temperature during high hydrostic pressure treatment is between –20°C and +100°C.
11. The method as claimed in claim 10, wherein at least one of the components of the peroxidase system is applied at least in the following concentrations: peroxidase, 0.01 pmol/ml hydrogen peroxide, thiocyanate, 0.01 µmol/ml chloride, 0.01 µmol/ml.
12. The method as claimed in claim 1 or 2, wherein: products or materials containing microorganisms are exposed to an amount of superatmospheric

pressure suitable for rendering said microorganisms more susceptible to inactivation by components of said peroxidase system.

13. The method as claimed in claim 12, to pasteurise, decontaminate or sterilise products or materials.
14. The method as claimed in claim 12, to pasteurise, decontaminate or sterilise food or feed.
15. The method as claimed in claim 12 to pasteurise, decontaminate or sterilise pharmaceutical preparations or cosmetics
16. The method as claimed in claim 12, wherein the level of superatmospheric pressure is between 100 and 1000 MPa, the exposure time to superatmospheric pressure is between 1 second and 5 hours, and the temperature during high hydrostic pressure treatment is between –20°C and +100°C
17. The method as claimed in claim 16, wherein at least one of the components of the peroxidase system is applied at least in the following concentrations: peroxidase, 0.01 pmol/ml hydrogen peroxide, thiocyanate, 0.01 µmol/ml chloride, 0.01 µmol/ml.
18. A composition containing components of a peroxidase system, comprising a peroxidase, a peroxidase substrate and hydrogen peroxide in an amount suitable to inactivate microorganisms, which have been exposed to a superatmospheric pressure.
19. The composition as claimed in claim 18, wherein hydrogen peroxide is produced by an oxidase in the presence of a suitable substrate for said oxidase.
20. The use of superatmospheric pressure and of a peroxidase enzyme, hydrogen peroxide and an oxidisable peroxidase substrate, to inactivate or kill microorganisms from the group consisting of bacteria, yeasts and fungi.
21. The use of superatmospheric pressure to render microorganisms from the group consisting of bacteria, yeasts and fungi more susceptible to inactivation by a peroxidase enzyme, hydrogen peroxide and an oxidisable peroxidase substrate.
22. An apparatus for killing or inactivating microorganisms, comprising: 1) operations of increasing pressure in an environment of microorganisms to a superatmospheric level; 2) operations of exposing said microorganisms to a peroxidase system

23. The apparatus as claimed in claim 22 further comprising sterility test tasks operable to generate and communicate messages to alter said pressure generating operations or said peroxidase system operations.
24. The apparatus of claim 22 or 23 to pasteurise, decontaminate or sterilise products or materials.
25. A process of enhancing the microbicidal activity of superatmospheric pressure, comprising: exposing microorganisms to a peroxidase catalysed oxidation reaction or to the oxidation products of said peroxidase catalysed oxidation reaction.
26. A process of enhancing the microbicidal activity of a peroxidase system, comprising: exposing the microorganisms to a superatmospheric pressure.

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Application No: GB 0025415.1

Examiner: Dr Fatema Sardharwala

Claims searched: 1-24

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Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
X	WO 98/48856 A1 (UNI OF ROCHESTER) see esp. line 36 of page 2 to line 10 of page 3 and Examples	1-24
X	DE 19649952 A1 (UHDE HOCHDRUCKTECHNIK) see esp. lines 6-16 of page 3, line 42 of page 3, line 62 of page 4 to line 18 of page 5	1-24
X	Applied and Environmental Biology, Vol 66, No. 10, 2000, C Garcia-Graells et al. "Inactivation of E Coli and L innocua in milk by combined treatment with high hydrostatic pressure and the lactoperoxidase system" pages 4173-4179	1-24

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